Photocrosslinking between nucleic acids and proteins: general discussion

Bhat, Vinayak; Cogdell, Richard; Crespo-Hernandez, Carlos E.; Datta, Ankona; De, Arijit; Haacke, Stefan; Helliwell, John; Improta, Roberto; Joseph, Joshy; Karsili, Tolga; Kohler, Bern; Krishnan, Retheesh; Mahil, L.; Lewis, Frederick; Mandal, Imon; Markovitsi, Dimitra; Mishra, Padmaja P.; Paul, Sneha; Periyasamy, Ganga; Pradeepkumar, P. I.

published in
Faraday Discussions
2018

DOI (link to publisher)
10.1039/c8fd90005a

document version
Publisher's PDF, also known as Version of record

Faraday Discussions

Cite this: *Faraday Discuss.*, 2018, **207**, 283

DISCUSSIONS

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Photocrosslinking between nucleic acids and proteins: general discussion

Vinayak Bhat, Richard Cogdell, Carlos E. Crespo-Hernández, Ankona Datta, Arijit De, Stefan Haacke, John Helliwell, Roberto Improta, Joshy Joseph, Tolga Karsili, Bern Kohler, Retheesh Krishnan, Mahil L, Frederick Lewis, Imon Mandal, Dimitra Markovitsi, Padmaja P. Mishra, Sneha Paul, Ganga Periyasamy, P. I. Pradeepkumar, Priyadarshi Roy Chowdhury, Manas Sarangi, Devika Sasikumar, Igor Schapiro, Gebhard F. X. Schertler, Ilme Schlichting, Javier Segarra-Martí, Rajaram Swaminathan, Vishnu V, Rienk van Grondelle, Reji Varghese and Ravindra Venkatramani

DOI: 10.1039/C8FD90005A

Tolga Karsili opened the discussion of the paper by Dimitra Markovitsi: It is well known that acidic substituents in aromatic systems contain $\pi\sigma^*$ states that are dissociative with respect to X–H bond fission (where X = N, O or S). It is also well known that the photochemistry of simple aromatic chromophores such as phenol and pyrrole are dominated by such states. In 9*H*-adenine, $\pi\sigma^*$ states have been predicted to give rise to N–H bond fission, localized on the amine moiety. In the AT duplex, the expected stabilization of the radical formed by N–H bond fission may lead to the stabilization of $\pi\sigma^*$ states. Such states have also been predicted to contain the initial signature of the solvated electron, formed by solute-to-solvent charge-transfer. Therefore, do you expect such states to dominate the observed initial N–H photodissociation in AT duplexes?

Dimitra Markovitsi answered: The role of the $\pi\sigma^*$ states in the photochemistry of nucleobases has been a subject of debate in the literature over the last few years. We stress that the N9–H bond of adenine involved in this state is accessible to water only for the bare nucleobase, and not for nucleosides or DNA multimers. The involvement of N6–H bonds can also be ruled out because we showed that base-pairing does not have an important effect on one-photon ionization.

Bern Kohler remarked: You observe adenine radicals that persist for milliseconds. What makes them relatively unreactive, and did you check to see whether the lifetimes depend on the nature or concentrations of the pH buffer components?

Dimitra Markovitsi responded: In the case of adenine single strands, we observed that replacing the phosphate buffer with a NaCl solution of the same ionic strength did not modify the lifetime of the deprotonated radicals.[1] It is worth noting that deprotonated guanine radicals produced by photosensitizers survive for hundreds of milliseconds;[2] the authors of that study stressed that hydrogen bonding decreases the radical lifetime, which is the opposite of our observations. A better knowledge of the final reaction products and possible intermediates is necessary in order to explain the observed kinetics.

1 A. Banyasz, T.-M. Ketola, A. Muñoz-Losa, S. Rishi, A. Adhikary, M. D. Sevilla, L. Martinez-Fernandez, R. Improta and D. Markovitsi, *J. Phys. Chem. Lett.*, 2016, **7**, 3949–3953.
2 Y. Rokhlenko, J. Cadet, N. E. Geacintov and V. Shafirovich, *J. Am. Chem. Soc.*, 2014, **136**, 5956–5962.

Carlos E. Crespo-Hernández asked: As you are aware, we have previously performed similar photoionization experiments for a wide range of monomers[1] and dinucleotides.[2] In those works, evidence was presented for the formation of hydrated electrons in monomers and dinucleotides through a combination of one- and two-photon absorption processes.

In the first study,[1] the observation of a one-photon ionization mechanism in the monomers at a photon energy of 4.66 eV (266 nm), which is much lower than their gas phase ionization energies (9 to 7.77 eV), was interpreted in terms of the effect that solvation has on the stabilization of the excited states of the monomers and the putative formation of hydrated electrons from either a charge-transfer-to-solvent state or *via* the photo-transfer of an electron in the excited molecule into a trap formed by favorably oriented molecular dipoles of water.[3,4] Do you think that a similar mechanism may be operative in the single- and double-stranded oligonucleotides that you have investigated in this and other recent works?

In our second study,[2] we observed that an increase in the concentration of the GpC, CpG, ApG and GpA dinucleotides in the range of 4 to 80 μM resulted in a systematic increase in the net photoionization yields (*i.e.* the combined one- and two-photon ionization yields), whereas no change in the photoionization yield was observed for the monomers within experimental uncertainty. Have you studied the dependence of the one- and two-photon ionization yields on the initial concentrations of the oligonucleotides that you have investigated? Such systematic investigations could potentially shed some light on the photoionization mechanism and the precursor state(s) leading to the one- and two-photon ionization of these systems.

1 C. E. Crespo-Hernández and R. Arce, *Photochem. Photobiol.*, 2002, **76**, 259–267.
2 C. E. Crespo-Hernández and R. Arce, *J. Phys. Chem. B*, 2003, **107**, 1062–1070.
3 A. L. Sobolewski and W. Domcke, *Chem. Phys. Lett.*, 2000, **329**, 130–137.
4 D. N. Nikogosyan, *Int. J. Radiat. Biol.*, 1990, **57**, 233–299.

Dimitra Markovitsi replied: Your papers have indeed been very stimulating for our studies. However, in our experiments, using much lower excitation intensities (<4 mW cm^{-2}) than yours (>4 mW cm^{-2}), we found that the one-photon ionization of mono-nucleosides and mono-nucleotides is lower than 3×10^{-4}, which corresponds to our detection limit. In contrast, when using similar excitation intensities to those reported in your 2002 paper we do observe ionization from the buffer. The fact that we observe one-photon ionization only for oligomers

exhibiting a pronounced secondary structure, in which nucleobases are, in principle, less exposed to the solvent compared to monomeric chromophores, does not corroborate the hypothesis that the interaction with water molecules is the dominant factor responsible for this phenomenon.

We did not vary the oligomer concentration because we optimized the experimental conditions in order to keep the excitation intensity as low as possible (<4 MW cm^{-2}). The best signal-to-noise ratio is obtained when the absorbance on the excitation side is $\sim$0.24 over 0.1 cm, which is used for all of our DNA systems.

Rajaram Swaminathan queried: What are the consequences of the ionisation of DNA due to low energy UV radiation inside the living cell?

Dimitra Markovitsi answered: One-photon ionization of DNA may be caused by solar light reaching the surface of the Earth (UVB). The resulting base radicals give rise to oxidative damage which is known to induce carcinogenic mutations.

Manas Sarangi asked: Why is the radical lifetime observed for double stranded AT longer than that found for polyadenine single strands?

Dimitra Markovitsi responded: Base pairing decreases the amplitude of the conformational motions of the system and, hence, the probability that the system adopts the configurations that are required for the radical reaction. This explanation is supported by two observations regarding the radical lifetime: (i) it is longer at lower temperatures (see Fig. 7b in our Faraday Discussions paper), and (ii) it is longer in the homopolymeric AT duplexes,[2] whose structures fluctuate less than the alternating AT duplexes.[1]

1 B. Bouvier, J.-P. Dognon, R. Lavery, D. Markovitsi, P. Millié, D. Onidas and K. Zakrzewska, *J. Phys. Chem. B*, 2003, **107**, 13512–13522.
2 A. Banyasz, T. Ketola, A. Muñoz-Losa, S. Rishi, A. Adhikary, M. D. Sevilla, L. Martinez-Fernandez, R. Improrta and D. Markovitsi, *J. Phys. Chem. Lett.*, 2016, **7**, 3949–3953.

P. I. Pradeepkumar questioned: Since G-quadruplex (G4) can form multiple topologies depending upon the sequence and salt conditions, what effect would these topologies have on the formation of radical cations? Furthermore, do you know which guanine base in G-quadruplex preferentially undergoes radical cation formation?

Dimitra Markovitsi replied: We are currently studying how the topology of the G-quadruplex and the type of cation that is present in the solution affect the formation and dynamics of radical cations. With regards to your second question, our experimental results do not provide information about the base that is responsible for electron ejection.

Ankona Datta asked: How do you characterize base pair dimerization in DNA? Does it make any difference to the structure of the DNA when the base pairs dimerize?

Dimitra Markovitsi answered: To the best of our knowledge, covalently linked dimers resulting from a photoreaction between Watson–Crick pairs have not been

characterized thus far. If such dimers are indeed formed, their effect on the conformation of the duplex should depend on their precise chemical structures.

John Helliwell communicated: In your paper, in the subsection "Reaction paths in $(AT)_1$ duplexes", distances of 2.6 Å and 2.0 Å are quoted. What are the standard uncertainties for these distances? This leads on to another question; what is the sensitivity of the authors' calculations to these distance values, *i.e.* what range of distance values must be allowed for due to error?

Dimitra Markovitsi communicated in reply: The accuracy of TD-DFT optimized geometries has been extensively benchmarked.[1] The errors in the distances compared to those obtained from wavefunction based methods depend on the type of bond and the nature of the optimized excited state, although they are typically $\sim 10^{-2}$ Å. Furthermore, we paid attention to select an appropriate functional (M052X) for the description of the Potential Energy Surface based on the character of our excited states. Thus, we are confident of the reported distances.

1 A. D. Laurent and D. Jacquemin, *Int. J. Quantum. Chem.*, 2013, **113**, 2019–2039.

Priyadarshi Roy Chowdhury communicated: Please could you discuss the conformational stability that is associated with the quadruplex formation?

Dimitra Markovitsi communicated in reply: G-quadruplexes were prepared by folding the corresponding single strand; the detailed protocol is described in our previous study.[1] Once formed, G-quadruplexes are quite stable at room temperature, as shown by their thermodynamic properties.[2] We also tested the stability of G-quadruplexes *versus* time-resolved experiments by checking their steady-state absorption spectra before and after laser irradiation.

1 A. Banyasz, L. Martínez-Fernández, C. Balty, M. Perron, T. Douki, R. Improta and D. Markovitsi, *J. Am. Chem. Soc.*, 2017, **139**, 10561–10568.
2 J.-L. Mergny, A.-T. Phan and L. Lacroix, *FEBS Lett.*, 1998, **435**, 74–78.

Tolga Karsili opened the discussion of the paper by Roberto Improta: Did you benchmark the energetics returned from the M052X functional against those returned from other Minnesota and long-range corrected functionals? Do you expect such benchmarking to reveal any significant changes in the reaction barriers for PCET?

Roberto Improta responded: We carried out several test calculations by employing both CAM-B3LYP and M06-2X functionals, for both the duplex and for an isolated Watson–Crick pair, and our conclusions are solid with respect to the choice of the functional.

Ravindra Venkatramani remarked: Your study addresses interstrand and intrastrand photoinduced CT processes in nucleobase stacks. However, the electronic states in such pi-stacks are expected to be delocalized over several nucleobases. What are your thoughts on photoinduced charge transfer and its mechanism in this context of strong electronic coupling between nucleobases?

Roberto Improta replied: I agree that the strong electronic coupling between the bases is an important factor for consideration. Actually, the decay paths involving charge transfer states, either intra- or inter-strand, are competitive with many other deactivation paths, both photophysical and photochemical. We explicitly tackled this issue in our previous work.[1] Since then, other possible decay paths have been recognized, involving both single (the excitation localizes on a single base and 'monomer like' decay is observed) and multiple bases (*e.g.* excimer minima or photodimerization paths).

1 R. Improta and V. Barone, *Angew. Chem., Int. Ed.*, 2011, **50**, 12016–12019.

Carlos E. Crespo-Hernández asked: In the absence of dynamics simulations, have you investigated the role of different base stacking conformations (such as base-stack roll, tilt, twist, rise, shift and slide) on the energy barriers that you calculated for the intra- and inter-strand proton coupled electron transfer (PCET) in the AT and GC systems that you have investigated? Do you think that different base stacking conformations can play a major role in the energetics and probabilities of participation of these relaxation pathways? Can you comment on the potential role of water molecules and counter ion interactions in modulating the rates and energetics (participation) of these different reaction pathways?

Previous experiments with AT and GC oligonucleotides and related systems (cited in your manuscript) have shown that the excited stacked complexes (often called excimers and exciplexes) in these systems overwhelmingly decay back to the ground state by charge recombination in tens of picoseconds. Have you investigated the mechanism of repopulation of the ground state in these systems after the PCET events?

Roberto Improta answered: The Charge Transfer (CT) processes, especially intrastrand CT between stacked bases, are surely affected by the stacking geometry of the bases and, therefore, by the conformational equilibria in the oligonucleotide. Such effects, which were investigated in a previous preliminary study on a GC model system,[1] are very important in a single strand. They also play a role in the duplex, although in this case our conclusion should be valid, at least for the structural family that we have investigated, which is representative of a GC repeat crystal structure. Interactions with water molecules are also likely to be less important for a duplex, whereas they should significantly modulate CT in a single strand, as discussed in a previous study.[2] In the two studies cited above we made some attempts to estimate the charge recombination rate, also including the vibrational effect,[1] based on Marcus theory, which gave promising results.

1 J. Cerezo, L. Martínez-Fernández, R. Improta and F. Santoro, *Theor. Chem. Acc.*, 2016, **135**, 221.
2 L. Martínez-Fernández, Y. Zhang, K. de La Harpe, A. A. Beckstead, B. Kohler and R. Improta, *Phys. Chem. Chem. Phys.*, 2016, **18**, 21241–21245.

Bern Kohler remarked: My question concerns the excited state pathway involving intrastrand electron transfer in the GC tetramer. Does the weak barrier that you observe between the two minima, corresponding to electron transfer with and without proton transfer, have implications for whether the PCET state is reached *via* concerted or sequential transfers? As you know, it has not yet been

possible to observe the formation kinetics of this state as it is formed faster than the experimental time resolution.

Roberto Improta responded: It is clear that only a purposefully tailored quantum dynamical study could unambiguously assess this issue. However, by looking only at the energetics, it seems likely that in the case of GC DNA the PCET process is sequential, whereas in other systems, such as AT DNA homo-polymer, it is concerted.

Vinayak Bhat queried: In your paper, you consider GC and AT DNA. I wonder whether proton-coupled electron transfer would be possible in DNA containing a mixture of GC and AT. For instance, if C1 and G2 in Scheme 1 were to be replaced with T1 and A1 respectively, would the $PCET^1$ pathway be favorable?

Roberto Improta replied: The intrastrand charge transfer processes would surely be affected by the nature of the adjacent bases, and a comparison between the ionization potentials and the electron affinities of the different bases, as well as their relative pK_a values, could provide a useful guide to help understand the possible reactions. With regards to $PCET$,[1] its energetics depend mainly on the Watson–Crick pair involved. On the other hand, the possibility of populating the inter-strand CT state will depend on the relative energy of this excited state with respect to the spectroscopic states, and on vibronic coupling with these latter states. As a consequence, the $PCET^1$ reaction can also be modulated by the nature of the stacked bases.

Rienk van Grondelle asked: I have a question about dynamical environmental effects; could you get a better look at PCET by taking into account some specific vibrations?

Roberto Improta answered: We plan to analyse the effect of vibrations, including the vibrational modes involving solvent molecules, in forthcoming studies. We have actually already gained some insight into the importance of vibronic effects for the charge transfer and charge recombination processes in stacked bases.[1–3] These studies demonstrated the role of vibrational modes in tuning the CT process. On the other hand, it is clear that for a fluctuating system as a single strand, where several energetics and vibrational parameters depend strongly on the conformation, it is not easy to take the interplay between slow and fast degrees of freedom into proper account. This latter point is an important challenge that could be tackled in the future.

1 J. Cerezo, L. Martínez-Fernández, R. Improta and F. Santoro, *Theor. Chem. Acc.*, 2016, **135**, 221.
2 F. Santoro, R. Improta, F. Avila, M. Segado and A. Lami, *Photochem. Photobiol. Sci.*, 2013, **12**, 1527–1543.
3 R. Improta, F. Santoro, V. Barone and A. Lami, *J. Phys. Chem. A*, 2009, **113**, 15346–15354.

Priyadarshi Roy Chowdhury communicated: Please can you discuss the role played by the dynamical environmental effects?

Roberto Improta communicated in reply: As extensively discussed in the paper, dynamical solvation effects strongly modulate the CT processes in DNA. First of all, full equilibration of the solvent degrees of freedom is important in order to stabilize all of the minima of the Charge Transfer (CT) states. Furthermore, the Proton Transfer (PT) reaction that occurs between two bases involved in an intra-strand CT state leads to a strong quenching of the excited state dipole (since it leads to a diradical 'neutral' state). Consequently, in this case, equilibration of the solvent degrees of freedom in the minimum of the CT state leads to an increase in the energy barrier associated with PT, and to a decrease in the driving force for this reaction.

Priyadarshi Roy Chowdhury communicated: Please can you also discuss the boundary conditions associated with the quantum mechanical calculations, which lead to photoactivated proton coupled electron transfer in DNA?

Roberto Improta communicated in reply: There are no particular boundary conditions included in our quantum mechanical calculations.

P. I. Pradeepkumar opened the discussion of the paper by Frederick Lewis:† How do you compare the effect of 7-deazadeoxyguanosine (ZdG) with that of dG in exciplex formation in hairpin DNAs?

Frederick Lewis answered: ZdG is a much better electron donor (lower oxidation potential) than dG and thus forms an exciplex with EG that has much more charge transfer character.

Rienk van Grondelle asked: Do you get real charge separation in the end in these structures?

Frederick Lewis responded: We think that EG-Z and EG2 form an exciplex and excimer, respectively, and exhibit extensive charge transfer character. However, the charges never separate since there is no other hole trap in these hairpins. We have studied hairpins that have EG and the hole trap Sd separated by two AT or GC base pairs. There is no evidence for charge separation in these hairpins.[1] Evidently, the decay of 1*EG is faster than hole transport to Sd.

1 K. E. Brown, A. P. N. Singh, Y.-L. Wu, A. K. Mishra, J. Zhou, F. D. Lewis, R. M. Young and M. R. Wasielewski, *J. Am. Chem. Soc.*, 2017, **139**, 12084–12092.

Stefan Haacke queried: Are the fluorescence spectra you show obtained after excitation at the lower end? How do they change when you excite in the oligomer bands at 250–260 nm? Is there energy transfer going on and the formation of excimer states?

Frederick Lewis replied: The fluorescence spectra shown were obtained with excitation at 350 nm. We did not determine fluorescence quantum yields for

† Frederick Lewis' paper was presented by Mahesh Hariharan, Indian Institute of Science Education and Research (IISER), Thiruvananthapuram, India.

250–260 nm excitation. I would expect them to be lower as a consequence of the competition between the energy transfer and nonradiative decay of the natural bases which would absorb most of the incident light, but the band shapes should be similar.

Retheesh Krishnan communicated: In your paper you mentioned that the excitonic coupling for EG-G is strong, whereas that for EG-C, -T and -A is weak. However the CD spectra for all of these look very similar. Can you please comment on this?

Frederick Lewis communicated in reply: You are correct that the CD excitonic coupling for EG-G is only slightly stronger than for EG-C, -T and -A. This is indicated by the red-shifted positive CD band at 295 nm, which is similar to that observed for EG-Z which demonstrates stronger exciton coupling than for EG-G.

Vishnu V communicated: This study essentially focuses on guanosine derivatives. Could the same study be carried out using derivatives of other nucleobases and, if so, do you think that the same results would be observed?

Frederick Lewis communicated in response: This is an interesting question. We looked for similar behavior in the cytosine derivative, pyrrolocytosine, but did not observe it.[1] The observation of strong exciton coupling requires the base derivatives to have strong electronic transition dipoles. This is not the case for most purines and pyrimidines.

1 P. P. Neelakandan, M. McCullagh, G. C. Schatz and F. D. Lewis, *J. Phys. Chem. B*, 2012, **116**, 5199–5204.

Priyadarshi Roy Chowdhury communicated: In the DNA structure, is the loop stability dependent on the unpaired bases that are present within the loop?

Frederick Lewis communicated in reply: Yes, the loop stability will depend on the number and identity of the unpaired bases. The TTT loop was selected because it does form stable hairpins with the base-paired stems employed in this study and does not serve as a potential electron donor. Other hairpins could have been used for this study.

Priyadarshi Roy Chowdhury communicated: How does timescale variation affect the studies associated with the structural aspects of DNA?

Frederick Lewis communicated in response: This is an excellent question. Geometric relaxation of the vertical excited state of EG, relaxation of the local duplex geometry with an increase in EG-purine stacking, and solvent relaxation could all be occurring on the timescales of the ps fluorescence and transient absorption decays. Unfortunately, time-resolved Raman is not sensitive to these changes.

Dimitra Markovitsi communicated: Can you compare the results obtained by fluorescence upconversion for your monomeric guanine analog EG-H_2 with those

obtained for the natural guanine nucleotide using the same experimental technique?[1]

1 F.-A. Miannay, T. Gustavsson, A. Banyasz and D. Markovitsi, *J. Phys. Chem. A*, 2010, **114**, 3256–3263.

Frederick Lewis communicated in reply: The excited state behavior of G has been studied in much greater detail than that of EG-H_2. Both additional experimental and computational studies similar to those conducted for G and vinyl G would have been desirable, but were not carried out prior to the end of our collaborative project. What we do know is that the lowest singlet of EG-H_2 is most likely of π,π^* character, is longer-lived, and has a higher fluorescence quantum yield than that of the lowest singlet(s) of G.

Rienk van Grondelle opened the general discussion of the papers by Dimitra Markovitsi, Roberto Improta and Frederick Lewis: What do you think is the overwhelming theme of this session?

Frederick Lewis replied: All three papers describe charge-transfer interactions between purine bases and adjacent bases. The first two papers deal with the duplexes of natural base pairs. The third paper deals with a base derivative (phenylethynylguanine) embedded in a hairpin. The unifying theme of the session is the role of these charge-transfer interactions in both the decay pathways and chemical reactions of duplex DNA.

Roberto Improta responded: I think that the importance of charge transfer processes in DNA is one of the main unifying themes of this session.

Dimitra Markovitsi answered: Although the three talks of this session reported different approaches to describe photo-induced processes in various DNA systems, the emerging common feature is the importance of charge transfer states. Such excited states could be responsible for electron ejection giving rise to base radicals that are observed on the millisecond timescale (see also my answer to Bern Kohler's question below). Quantum chemistry calculations showed how states with partial charge transfer character may decay *via* a mechanism involving proton coupled electron transfer. Finally, the charge transfer character of exciplexes in DNA hairpins containing non-natural bases was explored using ultrafast optical spectroscopy.

Rienk van Grondelle asked a general question: Why is nobody using EPR to detect radicals?

Bern Kohler replied: This is an interesting suggestion. EPR has of course been used to study radicals formed in DNA by ionizing radiation for many years, especially in low-temperature glasses where these radicals can be easily trapped. Charge recombination following UV excitation occurs efficiently on the picosecond timescale in DNA strands and is too fast to be observed by time-resolved EPR. However, it could be worth looking for any long-lived radicals that may have escaped geminate recombination.

Bern Kohler returned to the discussion of the paper by Dimitra Markovitsi: It is very interesting that you observe little to no photoionization of the monomeric bases under conditions that result in electron ejection from oligonucleotides. This would seem to point to the involvement of an excitation such as an excimer or CT excited state that is only formed in a DNA strand containing stacked bases. Your observation that the ionization yield is higher for your duplex at a lower temperature supports this notion, as a decreased temperature enhances the base stacking that is required for the formation of CT excited states. However, it is very difficult to understand how one-photon excitation could produce a CT state that then goes on to spontaneously lose an electron to the solvent, and so I am curious as to how you envision this possibility from an electronic structure standpoint? Indeed, it is difficult to imagine how an electron could be ejected from a nucleobase by photons with energies that are three eV below the gas-phase ionization potential of adenine. Furthermore, it would seem that this difficult task would be somewhat easier when adenine is fully solvated by water than when it is part of a base stack and exposed to a less polar environment, but you observe the opposite. It is much easier to imagine two-photon ionization in which a longer-lived excited state absorbs a second photon because the final energy is now greater than the gas-phase ionization potential.

Another comment concerns geminate recombination following one- *vs.* two-photon ionization. The greater final energy of the electron ejected by two-photon excitation should promote its escape from the field of its parent ion, whereas an electron released by one-photon ionization, which has little to no excess energy, should recombine efficiently. This suggests that even if one-photon ionization could take place with a high primary quantum yield, most electrons would decay by geminate recombination and would not be detected in nanosecond experiments. Can you comment on how you believe one-photon ionization to take place for bases in strands, but not for monomeric nucleobases?

Dimitra Markovitsi responded: Our working hypothesis is indeed that one-photon ionization is related to charge transfer states, which you have shown are formed in high yields upon low energy UV excitation of DNA multimers.[1] Although the great majority of these states decay rapidly, a small part of their population may undergo charge migration and charge separation.[2] These processes should be favoured in well-stacked base sequences. Conformational motions may prevent ion-pair recombination assisting electron ejection from the negatively charged base. According to this scenario, charge migration and charge separation are expected to have a more important role than the degree of exposure to solvent. It should be noted that the one-photon ionization quantum yields that we observe are extremely low ($\sim 10^{-3}$), thus making it very difficult to study the ionization and recombination of the ejected electron by ultrafast spectroscopy, as you have done for other systems.[3] Only purposefully tailored dynamical computational studies could assess this working hypothesis.

1 J. Chen, Y. Zhang and B. Kohler, *Top. Curr. Chem.*, 2015, **356**, 39–87.
2 D. B. Bucher, B. M. Pilles, T. Carell and W. Zinth, *Proc. Natl. Acad. Sci. U.S.A.*, 2014, **111**, 4369–4374.
3 J. Peon, G. C. Hess, J.-M. L. Pecourt, T. Yuzawa and B. Kohler, *J. Phys. Chem. A*, 1999, **103**, 2460–2466.

Rienk van Grondelle asked: If you can measure with pump–probe normal DNA, should the product not also be observable?

Dimitra Markovitsi answered: The final AT photodimers formed in genomic DNA have been characterized by analytical methods.[1] They cannot be identified by transient absorption spectroscopy because of the presence of many types of photolesions (the most important being pyrimidine dimers), which all absorb in the UV region.

1 S. Asgatay, A. Martinez, S. Coantic-Castex, D. Harakat, C. Philippe, T. Douki and P. Clivio, *J. Am. Chem. Soc.*, 2010, **132**, 10260–10261.

Carlos E. Crespo-Hernández queried : Can you comment on the potential effect that the temporal pulse width (*i.e.* ns *versus* fs) and repetition rate of the laser system may have on the one- and two-photon ionization yields for the DNA systems that you have investigated?

Dimitra Markovitsi responded: In our early study[1] we used 8 ns pulses with a repetition rate of 2 Hz. In our current experimental setup, which has an improved time-resolution (30 ns instead of 200 ns), the laser pulses are slightly shorter (5 ns) and the repetition rate is fixed to the lowest possible value (0.2 Hz), in order to avoid exciting transient species. However, the one-photon ionization quantum yields reported in our 2006 study and those presented in the current paper do not differ by more than 50%. More important differences are encountered in the two-photon ionization yields which, as highlighted also for other systems,[2] strongly depend on the experimental conditions.

1 S. Marguet, D. Markovitsi and F. Talbot, *J. Phys. Chem. B*, 2006, **110**, 11037–11039.
2 K. L. Stevenson, G. A. Papadantonakis and P. R. LeBreton, *J. Photochem. Photobiol. A*, 2000, **133**, 159–167.

Ravindra Venkatramani remarked: I wonder what the lower bound or threshold of the excitation energy would be to form a nucleobase radical. Would the absorption cross-sections at low energies matter for detecting such radicals?

Dimitra Markovitsi replied: Assuming that the one-photon ionization quantum yield for UVB excitation is the same as that determined at 266 nm, radical detection in transient absorption experiments should be possible if higher DNA oligomer concentrations (around 10^{-4} mol L^{-1}) are used.

Ganga Periyasamy returned to the discussion of the paper by Roberto Improta: The relationship between the number of base pairs and the conductivity has been discussed during the meeting. Similarly, is it possible to correlate the charge transfer/migration timescale with the number of DNA base pairs?

Roberto Improta answered: Although the CT processes that we have discussed mainly involve a pair of bases (either stacked or WC paired), charge transfer and charge migration processes are surely affected by the number of bases present in the polynucleotides, and this is critical for modulating the conformational

equilibria and the charge separation processes. The latter can significantly affect the reactivity of CT states.

Dimitra Markovitsi made a general comment: While the ns transient absorption technique has been available for several decades, only recently has it begun to be used to study chemical reactions triggered in DNA by the direct absorption of low energy UV radiation. These investigations have largely been stimulated by ultrafast spectroscopy and quantum chemistry studies which have provided important insights into excited state relaxation in DNA. Surprisingly, many of the UV-triggered reactions are very slow. New technical developments, which allow for the detection of very weak signals at times longer than 50 ms, could provide important information on biologically relevant processes. In this respect, it would be interesting to obtain inspiration from the original flash photolysis apparatus built by George Porter,[1] which I saw as a museum piece when I worked at the Davy-Faraday Research Laboratory, and combine it with modern electronic and optical devices.

1 G. Porter, *Nobel Lecture*, 1967, 1–23.

Rienk van Grondelle returned to the discussion of the paper by Roberto Improta: In my view if you look at, for example, a DNA duplex, charge transfer states are going to be present. Is there anyone who does not believe that the basic mechanism of creating DNA damage/radical states is *via* charge transfer states? The modern consensus on this now is amazing.

Roberto Improta responded: I agree, but it is important to remember that until a few years ago the involvement of CT states in DNA photoactivated dynamics was a hotly debated and challenged topic.

Dimitra Markovitsi opened the discussion of the paper by Javier Segarra-Martí: Can you please comment on the agreement of your calculated transient absorption bands in the UV and visible regions with those that have been experimentally determined for adenosine and $(dA)_{20}$?[1]

1 W.-M. Kwok, C. Ma and D. L. Phillips, *J. Am. Chem. Soc.*, 2006, **128**, 11894–11905.

Javier Segarra-Martí responded: The agreement is quite remarkable with the experimental pump–probe data of Kwok *et al.*,[1] where the main fingerprint is placed in the 300–360 nm probe window, which refers to our estimates in the 26–32k cm^{-1} probing window along Ω_3 and which we assign to the main excited state absorption signal featuring a doubly excited ($HOMO^2 \rightarrow LUMO^2$) character. This comparison validates the results obtained from our simulations and points towards the correct theoretical description of these high-lying and challenging electronic excited states. Moreover, the experimental 1D pump–probe data shows the difficulties faced by these experiments in resolving the differences between monomeric, dimeric and multimeric species when their photo-absorption signals overlap. It is in such cases where we believe multidimensional spectroscopies, such as those simulated in our paper, may provide new information that may be concealed in 1D pump–probe signals.

1 W.-M. Kwok, C. Ma and D. L. Phillips, *J. Am. Chem. Soc.*, 2006, **128**, 11894–11905.

Bern Kohler asked: In your spoken remarks, you referenced the 2DES experiments carried out by Prokhorenko *et al.*[1] Those authors reported both transient absorption and 2DES signals for adenine. Surprisingly, the former signals did not show evidence of the approximately 9 ps decay that is expected for the 7H tautomer of adenine, which accounts for 22% of the population in aqueous solution.[2] Did you simulate the 2DES spectra of the 7H and 9H tautomers of adenine, and are significant differences predicted?

1 V. I. Prokhorenko, A. Picchiotti, M. Pola, A. G. Dijkstra and R. J. Dwayne Miller, *J. Phys. Chem. Lett.*, 2016, **7**, 4445–4450.
2 B. Cohen, P. M. Hare and B. Kohler, *J. Am. Chem. Soc.*, 2003, **125**, 13594–13601.

Javier Segarra-Martí responded: We were aware of the mixture of 7H and 9H tautomers in adenine, which is why we resorted to working with the adenosine nucleoside. By doing this, we only needed to work with the 9H tautomer, which is the most biologically relevant and the tautomer present in DNA/RNA. We considered simulating 7H adenine but we expect some differences given their different lifetimes,[1] which should influence the broadenings observed in the 2D maps and would require us to work with a time dependent framework in order to clearly observe the differences between 7H and 9H adenine. 2DES measurements of adenine were reported by Moran and co-workers,[2] where they used 7-methyl and 9-methyl adenine to observe potential differences between the 7H and 9H tautomers. They observed shifts along the pump (Ω_1) frequency and were able to observe their different lifetimes, and also found that these particular energy shifts could also induce changes to the probe frequency (Ω_3), shifting the energetic position of the recorded absorption signals. If the main excited state absorption signals were different enough, one would be able to potentially differentiate between them. Otherwise one could also look at the broadening of the GSB and its time dependence, which would be similar to what has already been done with the 1D pump–probe technique,[1] in order to separate these two tautomers.

1 B. Cohen, P. M. Hare and B. Kohler, *J. Am. Chem. Soc.*, 2003, **125**, 13594–13601.
2 B. A. West, J. M. Womick and A. M. Moran, *J. Phys. Chem. A*, 2011, **115**, 8630–8637.

Rienk van Grondelle remarked: If you look at these ApA dimers, the two As are externally coupled. Why don't you see dimer/excitonic states in your spectra? If you look at the time dependence you should see an oscillatory phenomenon.

Javier Segarra-Martí responded: The time dependence (delay time) was set to zero in this particular study as we focused on analysing the excited state absorption signals that are characteristic of the Franck–Condon (equilibrium) region in order to understand which energy windows may be more convenient to monitor in upcoming experiments. If we included the time dependence we would indeed observe the oscillatory phenomenon mentioned in your question and which features in many other 2DES experiments due to the formation of excitons/excimers in close-lying and interacting absorbing moieties.

Stefan Haacke queried: When you compared different stacking interactions there seemed to be very little difference between the absorption spectra. If you sum them all up there would be little expectation of cross-peaks or differences in the excitation effect. Can you comment on this? Would you expect different lifetimes?

Javier Segarra-Martí answered: There is indeed very little difference in terms of the main absorption signatures, which is partially due to it being a homodimer (two adenine moieties) that absorbs at the very same pump (Ω_1) frequencies. I would expect more marked differences were you to consider heterodimers, as we observed in a recent study on adenine-uracil monophosphate (ApU),[1] given that adenine and uracil absorb at different Ω_1 and their fingerprints along the probe (Ω_3) frequency are also quite diverse. Moreover, these differences would be more marked when looking at delay times different from zero (considering a time dependence that is neglected in the manuscript), as the geometrical distortions driven along the excited state decay result in massive changes in the accessible high-energy levels and thus strongly modulate their position along Ω_3.[1] For the present case, different lifetimes could still be potentially extracted from specific regions of the spectra, particularly long-living decays arising due to intermolecular interactions (excimer formation) along Ω_1, which would in principle be well separated from the monomeric contributions that would show a smaller broadening along this particular dimension.

1 Q. Li, A. Giussani, J. Segarra-Martí, A. Nenov, I. Rivalta, A. A. Voityuk, S. Mukamel, D. Roca-Sanjuán, M. Garavelli and L. Blancafort, *Chem.–Eur. J.*, 2016, **22**, 7497–7507.

Roberto Improta asked: How do the qualitative features of the computed spectra depend on the details and 'level' of the CASPT2 calculation?

Javier Segarra-Martí replied: That is indeed a very good point, which we addressed in a previous study[1] for gas phase adenine. In that study we systematically addressed this point by increasing the active space size until convergence was reached at very large spaces featuring up to 12 additional π^* orbitals to increase the correlation. We then employed these reference values to build gas-phase dimers, similar to the systems under study (ApA) in our paper, and observed a generalised red-shift of the signals along both the Ω_1 (pump) and Ω_3 (probe) frequencies, which we recovered by employing an imaginary level shift as a semi-empirical correction of CASPT2 when too small active spaces must be used. Reduced active space approaches are required for large systems such as dimeric species (ApA) due to computational demands, and a qualitative agreement of the results with those employing larger active spaces can already be observed, with the quantitative agreement being improved with the aforementioned use of non-standard and calibrated imaginary level shift values.

1 A. Nenov, A. Giussani, J. Segarra-Martí, V. K. Jaiswal, I. Rivalta, G. Cerullo, S. Mukamel and M. Garavelli, *J. Chem. Phys.*, 2015, **142**, 212443.

Richard Cogdell opened the discussion of the paper by Padmaja P. Mishra: Could you please provide some more detail on how you hold onto the DNA on the

surface so that you can exert the force? Furthermore, when you look at individual molecules, how long does the fluorescence last? Is the effect reversible?

Padmaja P. Mishra replied: To hold the DNA on the surface and to apply force, we followed the experimental procedure that is described in the subsection "Annealing of Holliday junction and linker-DNA" in the Experimental section of our paper. In our experiments, PEG/Biotin-PEG-coated predrilled quartz microscope slides were used to monitor the fluorescence signals from the Cy3 and Cy5 labeled DNA substrate that was immobilized through biotin–streptavidin interactions.

We used a catalytic oxygen scavenging unit that consists of a mixture of protocatechuate 3,4-dioxygenase (PCD) and protocatechuic acid (PCA), which makes the fluorophores stable for 4–5 minutes. The process is not reversible, as once the fluorophores photobleach, they do not return to the original state.

Tolga Karsili remarked: In your quantum chemical calculations, a uniform field is applied to the zero-order electronic Hamiltonian. How do you choose the coordinate along which to position this field?

Padmaja P. Mishra replied: There seems to be some confusion here. There are no quantum calculations involved here. The field that is discussed here is a light induced optical force, which is applied at the Holliday junction arm in order to monitor the conformation Dynamics.

Arijit De addressed Padmaja P. Mishra and Tolga Karsili: I would like to rephrase Tolga's question. You focus the laser beam (along Z) to optically trap and hold a dielectric particle which is tethered to the single (protein) molecule by a linker, so that if you steer the laser beam focus in the focal plane (along X or Y) you pull the single molecule, therefore you apply a force to it. In which direction do you apply this force?

Padmaja P. Mishra responded: There seems to be some confusion with Tolga's Question. There are no quantum calculations involved here. The field that is discussed here is a light induced optical force, which is applied at the Holliday Junction arm in order to monitor the conformation Dynamics.

P. I. Pradeepkumar asked: What is the magnitude of the force exerted by the IHF on the 4WHJ in comparison to the applied external force? In other words, would it be possible to determine the force exerted by the IHF when it interacts with the 4WHJ?

Padmaja P. Mishra answered: Atomic force microscopy (AFM) based single-molecule force spectroscopy could be a suitable tool for quantifying the forces and binding mechanisms that lead to the formation of protein–DNA complexes. AFM and dynamic force spectroscopy are exciting tools that allow for the quantitative analysis of biomolecular interactions.

Arijit De queried: You described your results using sketches of Potential Energy Surfaces (PESs), with regard to how they are positioned relative to each other.

However, apart from estimating the force (in pN), what is also important (and perhaps more important) is the curvature of these PESs for estimating the force constant (in pN nm^{-1}) so that you know the trap stiffness. Have you measured force *vs.* distance curves in order to estimate the stiffness?

Padmaja P. Mishra replied: The position of the intermediates during branch migration (conformational fluctuation) is determined from the slope of the log-linear plot of K *vs.* applied force. For example, in our results the slope for K_f (1.04 nm, Fig. 2C) was found to be approximately 2.3 times higher than the slope for K_b (0.46 nm). This indicates that the distance of the *iso*I conformer from its TS is 0.58 nm further than that of the *iso*II conformer (Fig. 3, right panel). The distances represented here correspond to the separation between the X-R arms of the 4WHJ under different force conditions.

In our case, we have not estimated the force in these experiments. Instead, the force was calibrated using a parallel protocol, as described in the manuscript.

Rajaram Swaminathan questioned: Single molecule FRET experiments are not ensemble averaged, so can you please explain how you took care of noise related fluctuations in the data?

Padmaja P. Mishra responded: Photon trajectories from single molecule experiments usually report the structural changes and motions of biomolecules. We have used Hidden Markov Models (HMMs) that facilitate the extraction of the sequence of hidden states from noisy data through the construction of probabilistic models. Typically, the true number of states is determined by the Bayesian Information Criterion (BIC). However, constraints resulting from short data sets and Poisson-distributed photons in radiative processes such as fluorescence can limit the successful application of goodness-of-fit statistics. The HMM models used here enabled the unambiguous and unbiased separation of noise from state-to-state transitions and for a reliable analysis of noisy data, as well as enabling the examination and detection of significantly more complicated systems, including systems with multiple states, limited only by signal to noise. The molecule-by-molecule nature of the algorithm preserves one's ability to detect heterogeneities in the dynamics between molecules, which is critical in single-molecule studies. Potentially, the algorithm could also be used to discern states with the same FRET level but with different lifetimes.

Reji Varghese asked: Can you please comment on the dipole orientation of the donor and the acceptor?

Padmaja P. Mishra answered: The dipole orientation factor $\kappa 2$, which describes the relative orientation of the donor and acceptor transition dipoles, is an important parameter for FRET that ranges from 0 to 4. For example, when the donor and acceptor transition dipoles are aligned with each other, $\kappa 2 = 4$ and the orientation is ideal for energy transfer, whereas in a perpendicular orientation, $\kappa 2 = 0$, thus preventing any energy transfer regardless of the distance. It is important to realize that $\kappa 2$ matters only during the lifetime of the donor excited state when energy coupling is possible. For most fluorophores, this time is 1–10 ns. This means that if the fluorophores, their chromophore part, or the molecules

that the fluorophores are attached to move at frequencies that are significantly higher than 108–109 Hz, the fluorophore pair will experience a varying orientation while FRET occurs. In biological systems, proteins labelled with fluorophores can generally adopt a variety of conformations, and the covalent linkage between the fluorophore and the host protein can rotate. All of these factors lead to the randomization of the orientation, both spatially and temporally. Therefore, for most macromolecular interactions in solution, a $\kappa 2$ value of 2 or 3 is often assumed.

Minor polarizations of the donor and acceptor molecules will not lead to any major variations in the determination of R_0. However, if one assumes that a range of static donor–acceptor orientations are present that do not change during the lifetime of the excited state, a $\kappa 2$ value of 0.476 should be used instead. Nevertheless, under certain experimental conditions, such an assumption does not hold and so more rigorous treatments of $\kappa 2$ are called for.

Mahil L communicated: Why is the orientational stability restricted due to the binding of IHF with 4WHJs? What will happen to the DNA due to the binding of IHF?

Padmaja P. Mishra communicated in reply: The orientational stability that is restricted due to the binding of IHF with 4WHJs is a result of the imposed restriction on the 4WHJ conformational switching. HMM analysis of the time traces indicated a dramatic increase in the tightness of the 4WHJ upon binding to IHF, therefore reducing the distance between the arms. As IHF is a histone line protein, it usually bends the DNA upon binding. However, it should be mentioned that IHF is a sequence specific protein.

Vishnu V communicated: Will the length of the arms and their constituent nucleotides of the 4WHJ affect the force that is applied? I believe that the Holliday junction is only a small part of the sequence. On what basis is the rest of the sequence constructed?

Padmaja P. Mishra communicated in response: The length of the arms and their constituent nucleotides of the 4WHJ certainly affect the force that is applied. The conformational fluctuations of the arms also depend on the nucleotide sequence of the junction. The particular sequences that were used in this study were designed in such a way that, once annealed to the junction structure, the protein binding sequence will remain more towards the junction area. The rest of the sequence was randomly selected, while keeping in mind the necessity to have 50% purines and pyrimidines.

Sneha Paul opened the discussion of the paper by Bern Kohler: Is there any specific reason for choosing this particular deep eutectic solvent (glyceline)? The high viscosity of the solvent might make transient absorption studies difficult, and there are other less viscous deep eutectic solvents that are known, *e.g.* ethaline, that do not perturb the nucleic acids.

Bern Kohler replied: We were initially interested in glyceline on account of its high viscosity, which is nevertheless low enough to allow the solution to be

recirculated using a peristaltic pump. We have recently reported a time-resolved IR study of the $d(AT)_9 \cdot d(AT)_9$ duplex in ethaline (1 : 2 mol ratio of choline chloride and ethylene glycol).[1]

1 K. de La Harpe, F. R. Kohl, Y. Zhang and B. Kohler, *J. Phys. Chem. A*, 2018, **122**, 2437–2444.

Sneha Paul asked: Have you tried conducting these experiments in neat glycerol? If so, how different were the results?

Bern Kohler responded: We have not conducted experiments in neat glycerol. This is certainly feasible for the nucleobase monomers, but DNA strands are greatly destabilized in this solvent. Bonner and Klibanov showed that the melting temperature of a 22-mer double-stranded oligonucleotide with 52% GC content was lowered by approximately 30 °C (from 62 °C to 30 °C) upon going from a buffered aqueous solution to a glycerol solution.[1] Lower melting point AT-rich strands, such as those investigated in our study, are unlikely to retain the secondary structures of interest at room temperature in neat glycerol. The important point is that DNA strands are significantly more stable in a deep eutectic solvent made from choline chloride and an alcohol than they are in the alcohol alone.

1 G. Bonner and A. M. Klibanov, *Biotechnol. Bioeng.*, 2000, **68**, 339–344.

Carlos E. Crespo-Hernández queried: Given the estimation of a melting temperature in the range of 40–45 °C, reported in Fig. 4(c) and (d) of your manuscript for the double-stranded $d(AT)_9 \cdot d(AT)_9$ oligonucleotide in the deep eutectic solvent, what fraction of this oligonucleotide is present in a double stranded *versus* a single stranded form in the transient absorption experiments reported in Fig. 4(e) and (f) at room temperature?

Bern Kohler answered: In the two-state model of DNA melting, the absorbance *vs.* temperature curve provides a direct estimate of the fraction of folded DNA strands. From analysing the melting curve in Fig. 4(c), we estimate the fraction of intact duplexes at 20 °C to be greater than 90%.

Dimitra Markovitsi questioned: How do you subtract the signal that arises from two-photon ionization of the solvent? Do you consider that some of the exciting photons could be absorbed by DNA, therefore its intensity should be lower compared to that measured for the solvent alone?

Bern Kohler replied: Absorption by DNA (or by any absorbing solute for that matter) does reduce the total yield of solvated electrons that is formed by two-photon ionization of the solvent. For this reason, a transient absorption signal is recorded in a back-to-back measurement from a solution of adenosine 5′-monophosphate (AMP), which has an identical absorbance at the pump wavelength to that of the DNA solution of interest. This ensures that both solutions experience the same degree of two-photon excitation of the solvent. The AMP signal after 5 ps arises solely from solvated electrons and is used to predict the

signal contribution from these species at all delay times, as described in detail previously.[1]

1 C. E. Crespo-Hernández and B. Kohler, *J. Phys. Chem. B*, 2004, **108**, 11182–11188.

Rienk van Grondelle asked: Can you please expand on the effect of the solvent on inter-/intra-strand charge transfer? What is the timescale?

Bern Kohler responded: We have only made the first few steps towards understanding these solvent effects. Diverse measurements in aqueous solution make it very clear that CT states are only seen in strands made up of native nucleobases when the bases are closely stacked with each other. These deep eutectic solvents are virtually the only non-aqueous solvents that are known to stabilize DNA, so this is really the first opportunity to ask these kinds of questions. We have been unable to observe forward rates in any solvent thus far. Instead, the vibrational signatures of the CT states appear promptly within our time resolution. What have been amenable to study so far are the dynamics of charge recombination. Of course, even if the formation of CT states cannot be easily observed, it could be possible to reach conclusions about the effects of solvent on competing pathways such as inter- and intra-strand CT, if the yields of these channels can be estimated.

Ankona Datta questioned: Do you know the dielectric constant of the deep eutectic solvent that was used in this study?

Bern Kohler answered: Pandey *et al.*[1] estimated a value of 22 for glyceline from the measured Stokes shift of a solvatochromic probe molecule, but this was an indirect method. Their value is half that of neat glycerol, which has a static dielectric constant of 44 at 20 °C.[2]

1 A. Pandey, R. Rai, M. Pal and S. Pandey, *Phys. Chem. Chem. Phys.*, 2014, **16**, 1559–1568.
2 R. Behrends, K. Fuchs, U. Kaatze, Y. Hayashi and Y. Feldman, *J. Chem. Phys.*, 2006, **124**, 144512.

Roberto Improta remarked: I am curious about your results concerning the monomer. When you say that there is a two times slower decay, is the excited state lifetime twice as long or you are referring to the ground state recovery due to a slower vibrational cooling?

Bern Kohler replied: This statement refers to the excited state lifetimes as measured by the decay of excited-state absorption at a probe wavelength of 570 nm. Vibrational cooling also occurs more slowly in glyceline than in water, as is expected due to the former solvent's lower density of hydrogen bonds.

Joshy Joseph queried: I have a question regarding the photochemical stability of DNA in deep eutectic solvent systems. Since the generated excited states are much longer, will there be increased photo damage (*e.g.* thymine–thymine dimer formation) to DNA in these environments? What effect will the salt concentration have on the stability of the DNA duplex in deep eutectic solvent systems? Do

mono- and di-cationic salts exhibit the same role in stabilizing DNA in DESs as in aqueous systems?

Bern Kohler answered: To answer your first question, the photochemistry of DNA in deep eutectic solvents has not yet been investigated to the best of my knowledge. It would clearly be very interesting to do so. Although quite long-lived excited states are formed in glyceline (Fig. 3(d)), it is not clear whether these are suitable photoproduct precursors. Thymine–thymine dimers form in DNA in aqueous solution on an ultrafast time scale,[1] at least when excited near 260 nm. To answer your second question, 100 mM NaCl is often added to aqueous DNA solutions to stabilize duplex formation, but it is unnecessary to do this in deep eutectic solvents because the ionic components are already present in such high concentrations that there is plenty of dielectric screening. Of course, replacing the choline ion with something else could alter the stability, but this has not been explored very much yet.

1 W. J. Schreier, T. E. Schrader, F. O. Koller, P. Gilch, C. E. Crespo-Hernández, V. N. Swaminathan, T. Carell, W. Zinth and B. Kohler, *Science*, 2007, **315**, 625–629.

Ilme Schlichting remarked: In the previous session it was mentioned that structural dynamics have an influence that is difficult to probe. By using glyceline you are changing the electrostatics and viscosity. Have you considered using something like cellulose to change only the viscosity in order to see what effect the structural dynamics have?

Bern Kohler responded: It would be very interesting to isolate the effect of viscosity without changing the dielectric properties too much if this could be done without disrupting the structure of the DNA strand.

Imon Mandal communicated: Do the charges of the deep eutectic solvent (choline chloride) have any role in the stabilisation (modulation) of the CT states of the DNA duplex, except to change the polarity of the medium?

Bern Kohler communicated in reply: A number of properties beyond solvent polarity must be important when it comes to correctly describing the microscopic interactions in these solvents. After all, measurements of solvatochromic probe molecules in deep eutectic solvents have shown that they have similar polarities to those of the neat, hydrogen-bond donating alcohol solvents that they are made from.[1] Yet the latter solvents denature double-stranded DNA (or stabilize it only very weakly), while the deep eutectic solvents do not. In this initial study, we have invoked relatively simple and qualitative arguments about how, for example, the reduced solvent polarity expected in a deep eutectic solvent would affect parameters such as solvent reorganization energy that influence electron transfer rates. As a next step, additional time-domain spectroscopy and theory will be very important for understanding both the static and dynamic effects of deep eutectic solvents on photoinduced charge transfer reactions.

1 A. Pandey, R. Rai, M. Pal and S. Pandey, *Phys. Chem. Chem. Phys.*, 2014, **16**, 1559–1568.

Dimitra Markovitsi opened the general discussion of the papers by Javier Segarra-Martí, Padmaja P. Mishra and Bern Kohler, and asked Javier Segarra-Martí and Bern Kohler: Do you expect two-dimensional spectroscopy to provide better insight into UV-induced processes by studying DNA in deep eutectic solvents instead of aqueous solvents?

Javier Segarra-Martí answered: I would expect to be able to observe the differences between water and deep eutectic solvents more clearly through the specific inhomogeneous broadenings that they induce in the 2D signals. Water rearranges very quickly and produces a marked inhomogeneous broadening along the diagonal of the 2D peaks, whereas deep eutectic solvents are much more dense and I would expect their dynamics to be slower and thus provide a less pronounced effect in comparison. Additionally, and as Professor Kohler has discussed in his contribution, deep eutectic solvents appear to slow down the excited state decay, which would also reduce their homogeneous broadening with respect to the faster decays observed in water. By monitoring the shape of the peaks with 2DES and their evolution over time, one could potentially weight these two different contributions and potentially assess their relative importance.

Bern Kohler replied: I expect multidimensional ultrafast spectroscopy to reveal many new insights into DNA photophysics, no matter what solvent is used. There is already some exciting work in this direction, and there will be a great deal more as UV pulses with the requisite broad bandwidths become easier to generate.

Ravindra Venkatramani returned to the discussion of the paper by Bern Kohler: In addition to reorganization energy effects, the solvent can also electronically couple charge donor–acceptor states (the prefactor term in the Marcus rate expression). Can you comment on how the electronic coupling provided by the solvent may modulate the electron transfer rates in DNA duplexes (*e.g.* the forward ET for AT duplexes in your paper)?

Bern Kohler responded: We have not considered these effects as it has thus far been possible to satisfactorily describe ET rates using a very simple Marcus model in the few cases where this has been done (all in aqueous solution). It should be noted that it has not yet been possible to observe forward ET in UV-excited DNA strands as this appears to take place faster than the experimental time resolution.

Richard Cogdell returned to the discussion of the paper by Padmaja P. Mishra: I would like to ask more about the pulling of the force in your experiments. If you are pulling on a protein where there is a little flexibility with regards to where you pull, the moment of the force might be over a certain variability around where you actually measure. How do you account for that? How much elasticity is in your protein structure? Do you model it as stiff or elastic?

Padmaja P. Mishra answered: In our experiment, we did not pull the protein, rather we pulled one arm of the Holliday junction, while the protein is bound to the holiday junction. Details of the force calibration method and the experimental procedure for the application of force to the DNA holiday junction can be found in

the "Optical trapping and force calibration" subsection in the Experimental section of our paper.

P. I. Pradeepkumar returned to the discussion of the paper by Bern Kohler: Are there any crystal or NMR structure of duplex DNA or RNA reported in the presence of deep eutectic solvents? Is there any possibility of the formation of cation-pi interactions between the solvent and the nucleobases?

Bern Kohler replied: I am unaware of any experimental determinations of nucleic acid structures in deep eutectic solvents. Your second question about cation–pi interactions is very interesting. To my knowledge, this has not yet been discussed as a contributing factor towards stabilization. An atomistic simulation of the binding of the choline ion in the minor-groove of AT-DNAs showed that the hydroxy group of the choline ion interacts with the bases *via* hydrogen bonding.[1] The lower charge density of the choline cation compared with that of a monovalent or divalent metal ion also suggests that cation–pi interactions are likely to be weak. Finally, cation–pi interactions, at least those involving metal ions, have been suggested to stabilize unstacked conformations.[2] For these reasons, I do not expect cation–pi interactions to contribute significantly to the stabilization of nucleic acids in deep eutectic solvents.

1 M. Nakano, H. Tateishi-Karimata, S. Tanaka and N. Sugimoto, *J. Phys. Chem. B*, 2014, **118**, 379–389.
2 L. McFail-Isom, X. Shui and L. D. Williams, *Biochemistry*, 1998, **37**, 17105–17111.

Roberto Improta remarked: I have a couple of comments. (1) In these two sessions there has been a lot of focus on CT states, but we have not discussed the mechanisms explaining how they are populated. For example the monomer-like decay channel could be an important 'doorway' to inter-strand CT states, and if you increase the lifetime of the monomer-like decay channel (as happens in a deep eutectic solvent) this is going to affect all of the other channels.

(2) A significant part of the solvation effect is instantaneous, but there is an important contribution from a slow component, which in DNA could also coincide with a conformational rearrangement of the backbone structure, which could be made more difficult by the deep eutectic solvent. This suggests that the solvent dielectric constant is not the only parameter affecting the charge transfer pathways.

Bern Kohler replied: I couldn't agree more about the need to understand how CT states (both intra- and inter-strand) form in DNA strands. DNA is a complex, multichromophoric system and experiments have shown that UV excitation yields several distinct classes of excited states, which can differ in their spectroscopic signatures and dynamics. Many of the ultrafast laser experiments reported thus far for DNA strands are 'blind' to the dynamics that occur during the first few hundred femtoseconds, and it is in this brief window that the branching to the various decay channels likely takes place. It is also important to mention that CT states are not populated to any significant extent by direct photoexcitation at UV wavelengths where the DNA bases absorb strongly, such as at 260 nm. Consequently, the initially excited electronic states or excitons must rapidly evolve to CT

states, and this is perhaps influenced by the solvent. As we observe in our paper, the slower dynamical response of the deep eutectic solvent compared to that of water may affect how quickly CT states are formed, and may influence the competition between inter- and intra-strand channels, especially when these differ in charge transfer character. The ability to study the photophysics of DNA strands in non-aqueous environments makes it possible to systematically study effects such as these.

Rienk van Grondelle made a general comment: With multidimensional spectroscopy you are going to see every channel. If you could combine it with time resolved X-ray crystallography too, this could be a great tool for the future.

Gebhard F. X. Schertler responded: I think that further advances in multidimensional spectroscopy are very promising, and they will further aid in the interpretation of FEL measurements that are aimed towards a mechanistic explanation of quantum yields and stereoselectivity.

Devika Sasikumar returned to the discussion of the paper by Bern Kohler: This is interesting work Bern. Can you tell me whether the triplet state that is observed in 5′-TMP is merely due to the slow structural dynamics or due to any perturbation in the electronic states of the nucleobase? If I wished to populate the triplet state of an organic molecule, could a DES restrict the singlet modes that are responsible for fluorescence decay and cause intersystem crossing?

Bern Kohler responded: Nonradiative decay from an excited electronic state, whether by internal conversion or by intersystem crossing, always involves structural (nuclear) dynamics, so it may be difficult to disentangle the effects you mention. Triplet quantum yields for the pyrimidine nucleobase monomers increase as the solvent polarity decreases, even in solvents that are much less viscous than glyceline, and Crespo-Hernández and co-workers have made similar observations for 2-aminopurine.[1] These trends are explained by changes in the energies of nearby excited electronic states, but this of course results in changes in the nuclear dynamics.

1 C. Reichardt, C. Wen, R. A. Vogt and C. E. Crespo-Hernández, *Photochem. Photobiol. Sci.*, 2013, **12**, 1341–1350.

Rajaram Swaminathan asked: Is it possible to observe DNA–protein interactions and enzyme catalysis in deep eutectic solvents? Have such studies been reported?

Bern Kohler answered: A variety of enzymes have been shown to retain activity in deep eutectic solvents and catalyze synthetically valuable reactions.[1–6]

1 J. T. Gorke, F. Srienc and R. J. Kazlauskas, *Chem. Commun.*, 2008, **0**, 1235–1237.
2 D. Lindberg, M. de la Fuente Revenga and M. Widersten, *J. Biotechnol.*, 2010, **147**, 169–171.
3 P. Domínguez de María and Z. Maugeri, *Curr. Opin. Chem. Biol.*, 2011, **15**, 220–225.
4 V. Stepankova, S. Bidmanova, T. Koudelakova, Z. Prokop, R. Chaloupkova and J. Damborsky, *ACS Catal.*, 2013, **3**, 2823–2836.
5 Z.-L. Huang, B.-P. Wu, Q. Wen, T.-X. Yang and Z. Yang, *J. Chem. Technol. Biotechnol.*, 2014, **89**, 1975–1981.

6 H. Zhao, C. Zhang and T. D. Crittle, *J. Mol. Catal. B: Enzym.*, 2013, **85–86**, 243–247.

Reji Varghese asked: Is the cationic component of the eutectic mixture bound to the phosphate backbone of DNA? Could this be a possible way to dissolve DNA in organic solvents?

Bern Kohler responded: One study has suggested that choline ions have a high affinity for the minor groove of AT-rich DNAs where they form hydrogen bonds.[1] In my opinion, much remains to be learned about the behavior of DNA in deep eutectic solvents and its ultimate applications.

1 M. Nakano, H. Tateishi-Karimata, S. Tanaka and N. Sugimoto, *J. Phys. Chem. B*, 2014, **118**, 379–389.

Conflicts of interest

Gebhard F. X. Schertler is a co-founder of the leadXpro and InterAx biotechnology companies. There are no other conflicts to declare.